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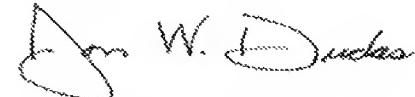
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APPLICATION NUMBER: 60/532,363

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This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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ENCLOSED APPLICATION PARTS (check all that apply)

- Specification Number of Pages 14
- Drawing(s) Number of Sheets 4
- Application Date Sheet. See 37 CFR 1.76

- CD(s), Number _____
- Other (specify) claims pages 2
Abstract 1page

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[Page 1 of 2]

Respectfully submitted,

SIGNATURE 

TYPED or PRINTED NAME Peter J. Phillips

Date December 24, 2003

REGISTRATION NO. 29,691

(if appropriate)

Docket Number: 0575-71126-Pro

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[Page 2 of 2]

Number 2 of 2

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Michael R. Rosen, Peter R. Brink, Ira S. Cohen and Richard B. Robinson
Serial No. : Not yet assigned
Filed : December 24, 2003
For : **CREATION OF A BIOLOGICAL ATRIOVENTRICULAR BYPASS TO COMPENSATE FOR ATRIOVENTRICULAR BLOCK**

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December 24, 2003

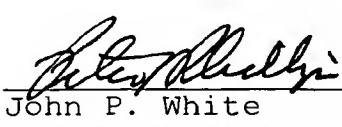
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*Application
for
United States Letters Patent*

To all whom it may concern

Be it known that

MICHAEL R. ROSEN PETER R. BRINK; IRA S. COHEN;
RICHARD B. ROBINSON;

have invented certain new and useful improvements in

CREATION OF A BIOLOGICAL ATRIOVENTRICULAR BYPASS TO
COMPENSATE FOR ATRIOVENTRICULAR BLOCK

of which the following is a full, clear and exact description

5 **CREATION OF A BIOLOGICAL ATRIOVENTRICULAR BYPASS TO COMPENSATE
FOR ATRIOVENTRICULAR BLOCK**

Statement of Federally Sponsored Research or Development

10 Work on this invention was sponsored by USPHS and NHLBI under award number HL-28958.

15 Background of the Invention

Throughout this application, various publications are referenced to as footnotes or within parentheses. Disclosures 20 of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citations for these references may be found at the end of this application, preceding the 25 claims.

One of the major indications for electronic pacemaker therapy is high degree heart block, such that a normally functioning sinus node impulse cannot propagate to the ventricle. The 30 result is ventricular arrest and/or fibrillation, and death.

Acute myocardial infarction (MI) afflicts millions of people each year inducing significant mortality and, in a large number of survivors, marked reductions in myocyte number and in 35 cardiac pump function. Adult cardiac myocytes divide only rarely, and the usual response to myocyte cell loss is hypertrophy that often progresses to congestive heart failure, a disease with a significant annual mortality. There have been recent reports of the delivery of mesenchymal stem cells 40 (MSCs a multipotent cell population of blood lineage) to the

5 hearts of post-Mi patients resulting in improved mechanical performance^{1,2}. The presumption in these and other animal studies³, is that the MSCs integrate into the cardiac syncytium and then differentiate into new heart cells restoring mechanical function.

10

5 Summary of the Invention

The present invention uses biological means for cell therapy to build a bypass tract in the heart that will take over the function of a diseased atrioventricular node. Adult human
10 mesenchymal stem cells (hMSCs) may be prepared in one of four ways (see below) and grown in culture on a non-bioreactive material. Once growth is complete the material has one end sutured to the atrium, and the other to the ventricle. Electrical signals generated by the sinus node to activate the
15 atria will propagate across the artificially constructed tract to excite the ventricle as well. In this way the normal sequence of atrioventricular activation will be maintained.

Four methods that may be used for preparing the hMSCs are:

20 1: In culture without incorporation of additional molecular determinants of conduction. Here the cells' own characteristic to generate gap junctions that communicate electrical signals are used as a means to propagate an
25 electronic wave from atrium to ventricle.

2: In culture following electroporation to add the gene for connexins 43, 40 and/or 45, the culture's electrotonic propagation of atrial signals to the ventricle.

30 3: In culture following electroporation to add the alpha and the accessory subunits of the L-type calcium channel, thereby increasing the likelihood of not just electrotonic propagation of a wavefront, but its active
35 propagation by an action potential.

4: A combination of 2 and 3.

5 The preparation of a bypass in this fashion not only will
facilitate propagation from atrium to ventricle, but will
provide sufficient delay from atrial to ventricular
contraction to maximize ventricular filling and emptying. The
goal is to mimic the normal activation and contractile
10 sequence of the heart. Moreover, this approach, when used
with gene therapy and stem cell technology to improve atrial
impulse initiation in the setting of sinus node disease offers
a completely physiologic system rather than its electronic
replacement.

15

According to the invention, a method of creating an
atrioventricular bypass tract for a heart is provided,
comprising growing mesenchymal stem cells into a strip with
two ends, attaching one end of the strip onto the atrium of
20 the heart, and attaching the other end of the strip to the
ventricle of the heart, to create a tract connecting the
atrium to the ventricle to provide a path for electrical
signals generated by the sinus node to propagate across the
tract and excite the ventricle.

25

5 Description of the Drawings

Figure 1. Identification of connexins in gap junctions of hMSCs. Immunostaining of Cx43 (**A**), Cx40 (**B**) and Cx45 (**C**). **D**, Immunoblot analysis of Cx43 in canine ventricle myocytes and 10 hMSCs. Whole cell lysates (120 μ g) from ventricle cells or hMSCs were resolved by SDS, transferred to membranes, and blotted with Cx43 antibodies. Migration of molecular weight markers is indicated to the right to the blot.

15 **Figure 2.** Macroscopic and single channel properties of gap junctions between hMSC pairs. Gap junction currents (I_{ij}) elicited from hMSCs using symmetrical bipolar pulse protocol showed two types of voltage dependent current deactivation: (A)-symmetrical, (B): asymmetrical.

20 **C,D** Single channel recordings from pairs of hMSCs. Pulse protocol (V_1 and V_2) and associated multichannel currents (I_z) recorded from a cell pair during maintained V_j of ± 80 mV. The discrete current steps indicate the opening and closing of single channels. Dashed line: zero current level. The all 25 points current histograms on the right-hand side revealed a conductance of ~ 50 pS. Glass coverslips with adherent cells were transferred to an experimental chamber perfused at room temperature (~ 22 °C) with bath solution containing (mM): NaCl, 150; KCl, 10; CaCl₂, 2; HEPES, 5 (pH 7.4); glucose, 5. 30 The patch pipettes were filled with solution containing (mM): K⁺ aspartate, 120; NaCl, 10; MgATP, 3; HEPES, 5 (pH 7.2); EGTA, 10 (pCa ~ 8); filtered through 0.22 μ m pores. When filled, the resistance of the pipettes measured 1-2 M Ω . Experiments were carried out on cell pairs using a double 35 voltage-clamp. This method permitted to control the membrane potential (V_m) and measure the associated junctional

5 currents (I_j).

Figure 3. Macroscopic properties of junctions in cell pairs between a hMSC and HeLa cell expressing only Cx40, Cx43 or Cx45. In all cases hMSC to Hela cell coupling was tested 6 to 12 hours after initiating co-culture.

10 **A,** I_j elicited in response to a series of voltage steps (V_j) in hMSC-HeLaCx43 pairs.

Top: symmetrical current deactivation; bottom:
asymmetrical current voltage dependence.

15 **B,** Macroscopic I_j recordings from hMSC-HeLaCx40 pairs exhibit symmetrical (top panel) and asymmetrical (bottom panel) voltage dependent deactivation.

20 **C,** Asymmetric I_j from hMSC-HeLaCx43 pair exhibits voltage dependent gating when Cx45 side is relative negative. I_j recorded from hMSC.

25 **D,** Cell-to-cell LY spread in cell pairs: from a HeLa Cx43 to an hMSC (top panel) and from an hMSC to a HeLa Cx43 to (bottom panel). In both cases a pipette containing 2 mM LY was attached to the left-handed cell in the whole-cell configuration.

30 Epifluorescent micrographs taken at 12 min after dye injection show LY spread to the adjacent (right-handed) cell. The simultaneously measured junctional conductance⁶ revealed g_j of ~16 nS and ~18 nS of the pairs, respectively. Cell Tracker green was used to distinguish 35 hMSCs from HeLa cells or vice versa in all experiments⁸.

Figure 4. Macroscopic and single channel properties of gap junctions between hMSC-canine ventricle cell pairs. Myocytes were plated between 12 and 72 hours and co-cultured with hMSCs for 6 to 12 hours before measuring coupling. **A, Top panel:** Phase-contrast micrograph of a hMSC-canine ventricle pair. **Bottom pane:** Monopolar pulse protocol (V_1 and V_2) and associated macroscopic junctional currents (I_z) exhibiting asymmetrical voltage dependence. **B, Top panel:** Multichannel current elicited by symmetrical biphasic 60 mV pulse. Dashed line, zero current level; dotted lines, represent discrete current steps indicative of opening and closing of channels. The current histograms yielded a conductance of ~ 40-50 pS. **Bottom panel:** Multichannel recording during maintained V_j of 60 mV. The current histograms revealed several conductances of 48 to 64 pS with several events with conductance of 84 pS to with 99 pS (arrows) which resemble operation of Cx43, heterotypic Cx40-Cx43 and/ or homotypic Cx40 channels.

5 Description of the Invention

According to the invention, a method of creating an atrioventricular bypass tract for a heart is provided, comprising growing mesenchymal stem cells into a strip with
10 two ends, attaching one end of the strip onto the atrium of the heart, and attaching the other end of the strip to the ventricle of the heart, to create a tract connecting the atrium to the ventricle to provide a path for electrical signals generated by the sinus node to propagate across the
15 tract and excite the ventricle.

The steps of attaching may be performed by suturing. The stem cells may be adult human mesenchymal stem cells. The step of growing may comprise growing the stem cells in culture on a
20 non-bioreactive material. The step of growing may be performed in an environment substantially free of any additional molecular determinants of conduction.

The method may further comprise a step of adding a gene to the
25 mesenchymal stem cells by electroporation. The gene may encode for a connexin, such as connexin 40, connexin 43, and/or connexin 45. The step of adding a gene by electroporation may include adding alpha and accessory subunits of L-type calcium. The step of adding a gene by
30 electroporation may include adding the gene for connexins and adding alpha and accessory subunits of L-type calcium channel.

MSCs express connexins that are the building block proteins of
35 gap junctions and can form functional gap junctions with one another, with cell lines expressing cardiac connexins, and with adult cardiac myocytes. Further, the connexins expressed

5 suggest that hMSCs should readily integrate into electrical syncytia of many tissues promoting repair or serving as the substrate for a therapeutic delivery system.

Human mesenchymal stem cells (Poietics™ hMSCs - Mesenchymal
10 stem cells, Human Bone Marrow) were purchased from Clonetics/BioWhittaker (Walkersville, M.D.) and cultured in MCS growing media and used from passages 2-4. Typical punctate staining for Cx43 and Cx40 was seen along regions of intimate cell to cell contact of the MSCs grown in culture as
15 monolayers (Figure 1 A,B). Cx45 staining was also detected but unlike that of Cx43 or Cx40 was not typical of connexin distribution in cells. Rather it was characterized by fine granular cytoplasmic and reticular-like staining with no readily observed membrane associated plaques (Figure 1C).
20 This does not exclude the possibility that Cx45 channels exist but does imply that their number relative to Cx43 and Cx40 homotypic, heterotypic and heteromeric channels is low. Figure 1D illustrates Western blot analysis⁴ for canine ventricle myocytes and hMSCs with a Cx43 polyclonal antibody
25 which adds further proof of Cx43 presence in hMSCs.

Gap junctional coupling among hMSCs is demonstrated in Figure 2. Junctional currents recorded between hMSC pairs show quasi-symmetrical (Figure 2A) and asymmetrical (Figure 2B) voltage dependency arising in response to symmetric transjunctional voltage steps of equal amplitude but opposite sign. These behaviors are typically observed in cells which co-express Cx43 and Cx40⁴.

35 Figures 2C and 2D illustrate typical multichannel recordings from a hMSC pair. Using 120 mM K aspartate as a pipette solution channels were observed with unitary conductances of

5 28-80 pS range. Operation of channels with ~50 pS conductance (see Figure 2 C) is consistent with previously published values^{5,6} for Cx43 homotypic channels. This does not preclude the presence of other channel types, it merely suggests that Cx43 forms functional channels in hMSCs.

10

To further define the nature of the coupling hMSCs were co-cultured with human HeLa cells stably transfected with Cx43, Cx40, and Cx45⁷ and it was found that hMSCs were able to couple to all these transfectants. Figure 3A shows an example of junctional currents recorded between an hMSC and HeLaCx43 cell pairs that manifested symmetrically and asymmetrically voltage dependent currents. The quasi-symmetric record suggests that the dominant functional channel is homotypic Cx43 while the asymmetric record suggests the activity of another connexin in the hMSC (presumably Cx40 as shown by immunohistochemistry, see Figure 1) that could be either a heterotypic or heteromeric form or both. These records are similar to those published for transfected cells: heterotypic and mixed (heteromeric) forms of Cx40 and Cx43^{4,8}. Co-culture of hMSCs with HeLa cells transfected with Cx40 (Figure 3B) also revealed symmetric and asymmetric voltage dependent junctional currents consistent with the co-expression of Cx43 and Cx40 in the hMSCs similar to the data for Cx43 HeLa-hMSC pairs. HeLa cells transfected with Cx45 coupled to hMSCs always produced asymmetric junctional currents with pronounced voltage gating when Cx45 (HeLa) side was negative (Figure 3C). This is consistent with the dominant channel forms in the hMSC being Cx43 and Cx40 as both produce asymmetric currents when they form heterotypic channels with Cx45^{4,8}. This does not exclude Cx45 as a functioning channel in hMSCs but it does indicate that Cx45 is a minor contributor to cell to cell coupling in hMSCs.

5 The lack of visualized plaques in the immunostaining for
Cx45 (Figure 1) further supports this interpretation.

Figure 3D shows Lucifer Yellow transfer from HeLaCx43 cell
to an hMSC cell (top panel) and transfer from an hMSC to a
10 HeLaCx43 (bottom panel). The junctional conductance of the
cell pairs was simultaneously measured by methods described
earlier⁶ and revealed conductances of ~ 16 nS and ~18 nS,
respectively. The transfer of Lucifer Yellow was similar to
that previously reported for homotypic Cx43 or co-expressed
15 Cx43 and Cx40 in HeLa cells⁶. Cell Tracker green (Molecular
Probes) was always used in one of the two populations of
cells to allow heterologous pairs to be identified⁸.

hMSCs were also co-cultured with adult canine ventricular
20 myocytes. As shown in Figure 4 the hMSCs couple electrically
with cardiac myocytes. Both macroscopic (Figure 4A) and
multichannel (Figure 4B) records were obtained. Junctional
currents in Figure 4A are asymmetric while those in Figure
25 4B show unitary events of the size range typically resulting
from the operation of homotypic Cx43 or heterotypic Cx43-
Cx40 or homotypic Cx40 channels^{4,8}. Heteromeric forms are also
possible whose conductances are the same or similar to
homotypic or heterotypic forms.

30 In studies of cell pairs were demonstrated effective
coupling of hMSC to other hMSC (13.8 ± 2.4 nS, n=14), to HeLa
Cx43 (7.9 ± 2.1 nS, n=7), to HeLa Cx40 (4.6 ± 2.6 nS, n=5), to
HeLa Cx45 (11 ± 2.6 nS, n=5) and to ventricular myocytes
35 (1.5 ± 1.3 nS, n=4). Results show that hMSCs couple to one
another via Cx43 and Cx40. In addition, they form functional
gap junction channels with cells transfected with Cx43, Cx40
or Cx45 as well as canine ventricular cardiomyocytes. These

5 data support the possibility of using MSCs as a therapeutic
substrate for repair of cardiac tissue. Other syncytia such
as vascular smooth muscle or endothelial cells should also
be able to couple to the hMSCs because of the ubiquity of
Cx43 and Cx40^{9,10}. Thus they may also be amenable to hMSCs
10 based therapeutics, as follows: hMSCs can be transfected to
express ion channels which then can influence the
surrounding syncytial tissue.

Alternatively, the hMSCs can be transfected to express genes
15 that produce small therapeutic molecules capable of
permeating gap junctions and influencing recipient cells.
Further, for short term therapy, the small molecules can be
directly loaded into hMSCs for delivery to recipient cells.
The success of such an approach is dependent on gap junction
20 channels as the final conduit for delivery of the
therapeutic agent to the recipient cells. The feasibility of
one such approach was demonstrated by transfecting hMSCs
with mHCN2, a gene encoding the cardiac pacemaker channel,
and delivering them to the canine heart where they generate
25 a spontaneous rhythm.

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5 We claim:

1. A method of creating an atrioventricular bypass tract for a heart, comprising:

growing mesenchymal stem cells into a strip with two ends;

attaching one end of the strip onto the atrium of the heart, and

attaching the other end of the strip to the ventricle of the heart, to create a tract connecting the atrium to the ventricle to provide a path for electrical signals generated by the sinus node to propagate across the tract and excite the ventricle.

2. The method of claim 1, wherein the steps of
attaching are performed by suturing.

3. The method of claim 1, wherein the stem cells are adult human mesenchymal stem cells.

25 4. The method of claim 3, wherein the step of growing
comprises growing the stem cells in culture on a non-
bioreactive material.

30 5. The method of claim 4, wherein the step of growing
is performed in an environment substantially free of any
additional molecular determinants of conduction.

6. The method of claim 1, further comprising a step of adding a gene to the mesenchymal stem cells by electroporation.

7. The method of claim 6, wherein the gene encodes for a connexin.

10 8. The method of claim 7, wherein the connexin includes connexin 40.

9. The method of claim 7, wherein the connexin includes connexin 43.

15 10. The method of claim 7, wherein the connexin includes connexin 45.

20 11. The method of claim 6, wherein the step of adding a gene by electroporation includes adding alpha and accessory subunits of L-type calcium.

25 12. The method of claim 6, wherein the step of adding a gene by electroporation includes adding the gene for connexions and adding alpha and accessory subunits of L-type calcium channel.

5 **CREATION OF A BIOLOGICAL ATRIOVENTRICULAR BYPASS TO COMPENSATE
FOR ATRIOVENTRICULAR BLOCK**

Abstract of the Disclosure

A method of creating an atrioventricular bypass tract for a
10 heart comprises growing mesenchymal stem cells into a strip
with two ends, attaching one end of the strip onto the atrium
of the heart, and attaching the other end of the strip to the
ventricle of the heart, to create a tract connecting the
atrium to the ventricle to provide a path for electrical
15 signals generated by the sinus node to propagate across the
tract and excite the ventricle.

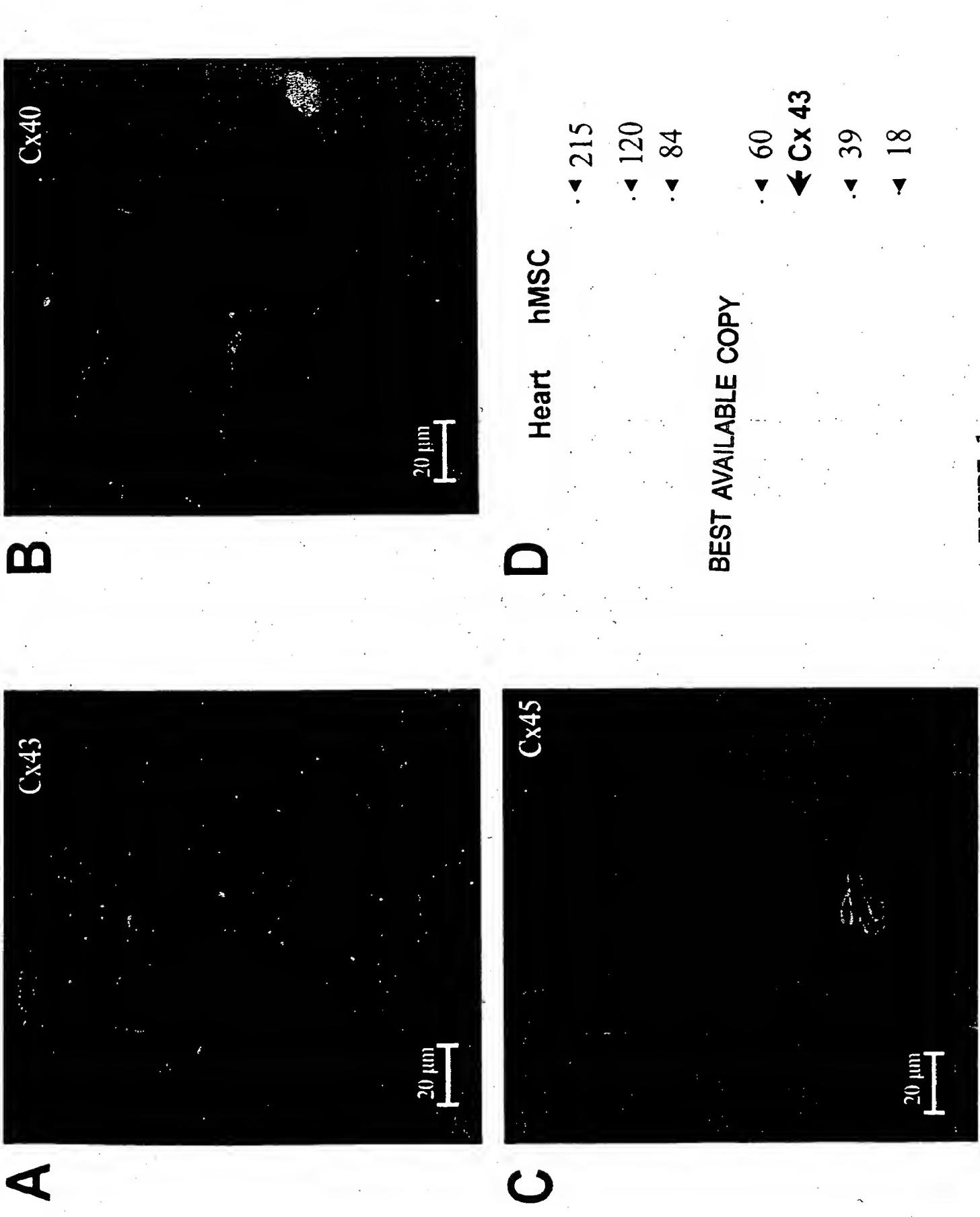


FIGURE 1

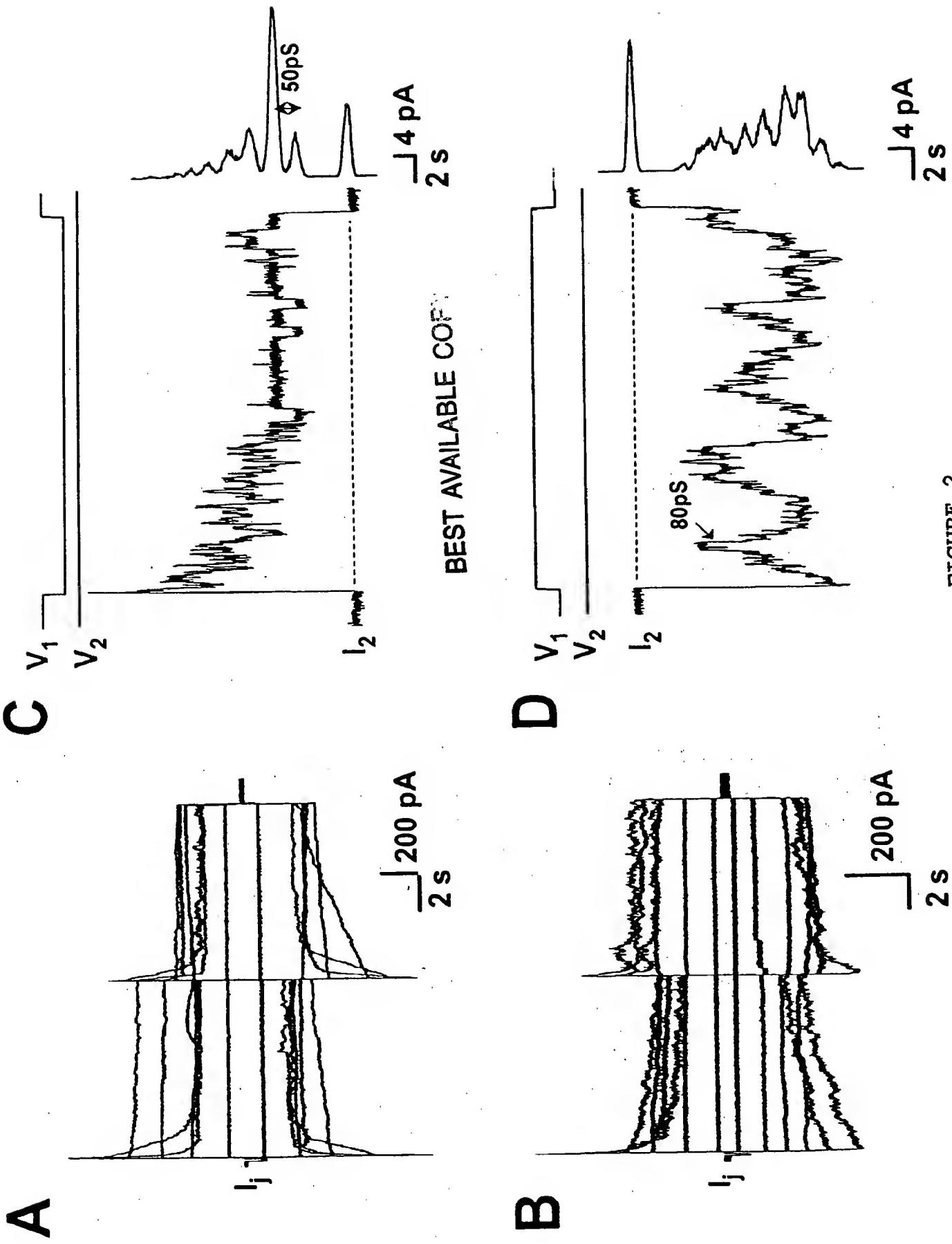
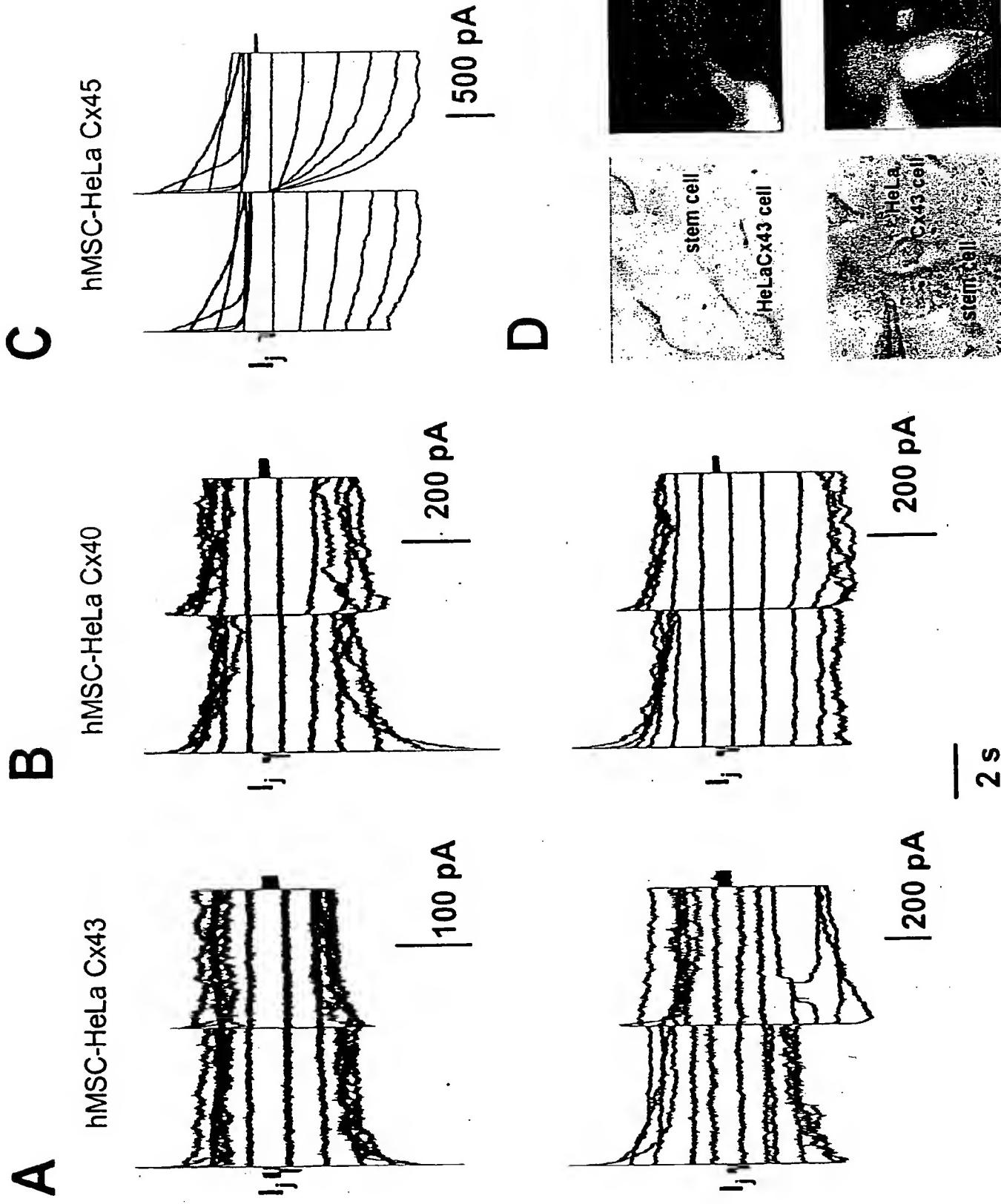


FIGURE 2

BEST AVAILABLE CO₂

FIGURE 3



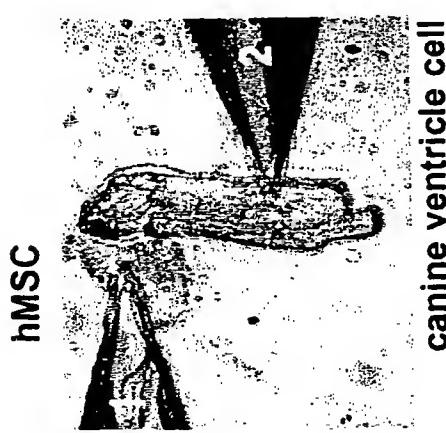
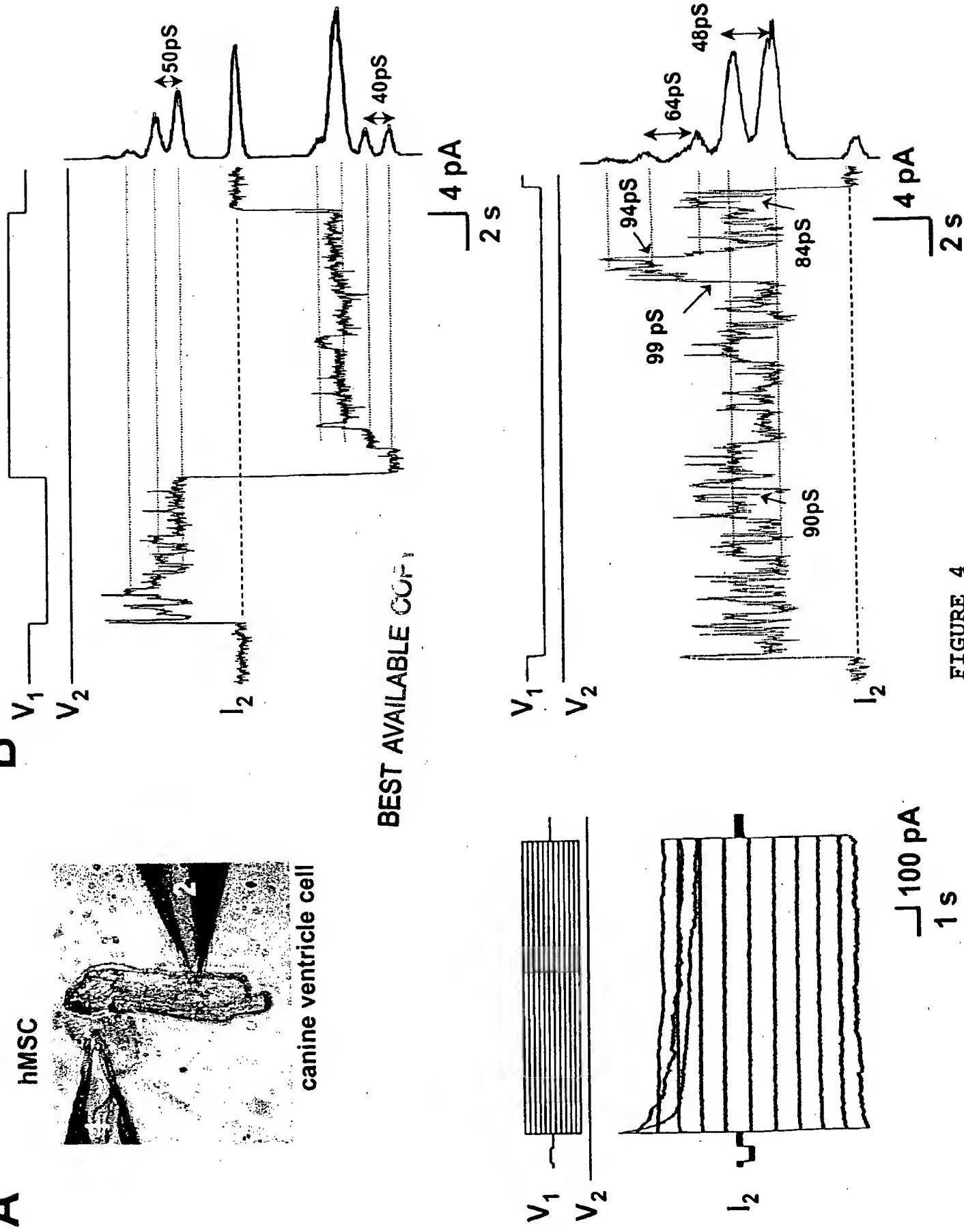
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FIGURE 4